On page 11, line 10, after "sequences", insert --Cotton fiber transcriptional initiation regions chosen for cotton fiber modification may include the 4-4, rac13 and Ltp cotton fiber promoter regions provided herein.--.

D3

On page 27, between lines 14-15, insert the following:

--Color phenotypes can be assessed by the use of a colorimeter, an instrument which is already used to provide objective measurements of the color of cotton samples. A colorimeter uses a combination of light sources and filters to make various estimates of samples of colors, sometimes referred to as tristimulus values.

In the past such estimates have been used to calculate a value (Hunter's +b, described below) indicating the degree of yellowness of a cotton sample. The yellowness and reflectance (from Rd, the degree of lightness of darkness of the samples) has been used to provide cotton color measurements for grading. Tests are typically conducted by exposing the face of a sample to a controlled light source. A typical color chart showing how the official grade standards relate to Rd and +b measurements is shown in *Cotton*, RJ Kohel and CF Lewis, Eds., #24 in AGRONOMY Series-American Soc. Agronomy (see Figure 12-6 therein).

D4

Various colorimeter methods can be so used to quantify color and express it numerically. The Munsell method, devised by the American artist A. Munsell, uses a classification system of paper color chips assorted according to their hue (Munsell Hue), lightness (Munsell Value), and saturation (Munsell Chroma) for visual comparison with a specimen color.

Other methods for expressing color numerically have been developed by an international organization concerned with light and color, the Commission Internationale de l'Eclairage (CIE), having a Central Bureau located at Kegelgasse 27, A-1030 Vienna, Austria. The two most widely known of these methods are the Yxy color space, devised in 1931 based on the tristimulus value XYZ, as defined by CIE, and the L*a*b* color space, devised in 1976 to provide more uniform color differences in relation to visual differences. Color spaces* such as these are now used throughout the world for color communication. The Hunter Lab

81

D

color space was developed in 1948 by R.S. Hunter as a uniform color space which could be read directly from a photoelectric colorimeter (tristimulus method).

D4

The L*C*h color space uses the same diagram as the L*a*b* color space, but uses cylindrical coordinates instead of rectangular coordinates. In this color space, L* indicates lightness and is the same as the L* of the L*a*b* color space, C* is chroma, and h is the hue angle. The value of chroma C is 0 at the center and increases according to the distance from the center. Hue angle is defined as starting at the +a axis of the L*a*b* space, and is expressed in degrees in a counterclockwise rotation. Thus, relative to the L*a*b* space, 0° and 360° would be at the +a* line, 90° would be the +b*, 180° would be -a* and 270° would be -b*. All of the above methods can be used to obtain precise measurements of a cotton fiber color phenotype.--.

On page 28, line 8, change "lZapII" to --λZapII--;

Line 22, change "4^{O}C" to --4°C--;

Line 24, change "20^{O}C" to --20°C--;

Line 25, change "4^{O}C" to --4°C--.

On page 29, line 7, change "100mMEDTA" to --100mM EDTA--;

Line 9, change "65^{O}C" to --65°C--.

On page 30, line 8, change "42^{O}C" to --42°C--.

On page 33, between lines 12-13, insert the following:

D5

--The #105 lipid transfer protein cDNA clone was used as a probe against cotton tissue and in a cotton fiber northern. The northern showed that the cotton fiber Ltp is highly expressed in cotton fiber. The mRNA that codes for this protein is expressed throughout fiber development at extremely high levels. Northern blots indicate that this mRNA is expressed at 5 dpa and is continually expressed at a high level at 40 dpa.--.

On page 33, after line 20, insert the following:

--The cotton genomic library was probed with a 3'-specific Ltp probe and 6 genomic phage candidates were identified and purified. Figure 7 provides an approximately 2 kb sequence of the Ltp promoter region which is immediately 5' to the Ltp encoding region.

D6

Six genomic phage clones from the cotton genomic library were identified using a 3'-specific probe for the Ltp mRNA. This was done to select the promoter from the Ltp gene that is maximally expressed in cotton fiber from the family of Ltp genes in cotton. The Ltp promoter is active throughout the fiber development period.--.

On page 37, line 19 change "PCR'ed" to --PCR amplified--.

On page 39, between lines 3-4, insert the following:

--Melanin

A binary construct for plant transformation to express genes for melanin synthesis is prepared as follows. The melanin genes were originally isolated from the common soil bacterium *Streptomyces antibioticus* (Bernan, *et al. Gene* (1985) *37*:101-110). Melanin production is composed of a two gene system. The first gene, *tyr*A, encodes the catalytic unit responsible for the polymerization of the amino acid tyrosine, the primary substrate, and is termed tyrosinase. The second gene, ORF438, is responsible for binding copper and delivering copper to the tyrosinase and activating the enzyme. Expression of both the ORF438 and *tyr*A genes ensures maximal tyrosinase activity.

D7

The genes for both ORF438 and *tyr*A were fully re-synthesized with respect to their DNA sequence. This was performed as the initial DNA sequence isolated from *Streptomyces* has a very high guanine and cytosine (G+C) DNA content. Thus, the ORF438 and *tyr*A genes were re-synthesized to appear more "plant-like" (reduced G+C content) with respect to plant preferred codons encoding their corresponding amino acids.

The re-synthesized ORF438 and *tyr*A genes were treated in two distinct ways depending on which compartment in the fiber cell the final protein products would be localized. One chimeric gene/plant binary construct (designated pCGN5148) contained the genes targeted to the fiber cell plastids. To do this, 12 amino acids of a gene for the small

subunit of carboxylase (SSU) plus the original 54 amino acid SSU transit peptide were fused to the amino termini of both the ORF438 and *tyr*A gene products respectively. These peptide sequences allow the ORF438 and *tyr*A gene products (proteins) to be efficiently targeted to the plastid. This targeting was initiated as the plastid is the site of tyrosine production within the fiber cell.

The second chimeric gene/plant binary construct (designated pCGN5149) contained the ORF438 and *tyr*A genes targeted to the vacuole within the fiber cell. Based on information from other biological systems, it was postulated that the fiber cell vacuole may contain a high concentration of tyrosine for melanin polymerization. Both the ORF438 and *tyr*A genes contain the 29 amino acid signal peptide from a tomato carboxypeptidase inhibitor (CPI) protein as amino terminal gene fusions to direct these proteins to the endoplasmic reticulum (ER) secretory system of the fiber cell.

In addition, the *tyr*A gene has an 8 amino acid vacuolar targeting peptide (VTP) from CPI fused at the carboxy terminus so that the mature copper-activated tyrosinase will eventually be targeted to the vacuole of the fiber cell. Both the ORF438 and *tyr*A proteins also had potential glycosylation sites removed via site-directed mutagenesis of the ORF438 and *tyr*A genes, respectively. Potential plant cell glycosylation of these proteins upon their expression in fiber cells could result in tyrosinase inactivation, hence removal of potential glycosylation sites was deemed necessary.

The modified genes for both the plastid and vacuolar targeted ORF438 and tyrosinase proteins were placed into a fiber expression cassette to be "switched" on during development of the cotton fiber cell. The "switch" (promoter) utilized for the melanin constructs was 4-4. The modified ORF438 and *tyr*A genes were cloned into the 4-4 promoter cassette and these chimeric genes then inserted into a binary plasmid to create plasmids pCGN5148 and pCGN5149, containing the modified genes for plastid and vacuolar targeted ORF438 and tyrosinase proteins, respectively. These binary plasmids also contain genetic determinants for their stable maintenance in *E. coli* and *Agrobacterium* and also contain a chimeric gene for

plant cell expression of the bacterial kanamycin resistance gene. This kanamycin resistance marker allows for the selection of transformed versus non-transformed cotton cells when plant hypocotyl or leaf segments are infected with *Agrobacterium* containing the binary plasmids.

A block diagram of the plasmid pCGN5149, having vacuolor targetting sequences, is shown in Figure 8. Plasmid pCGN5148 (not shown) is constructed the same as pCGN5149; only pCGN5148 has plastid-targeting sequences.

Indigo

D7

Indigo production involves conversion of the amino acid tryptophan, the primary substrate, into indole which is then converted into indoxyl. Molecules of indoxyl spontaneously convert to indigo in the presence of oxygen. A two gene system was used to effect indigo production in fiber cells. The first gene (*tna*) was obtained from the bacterium *E. coli* and encodes the enzyme tryptophanase. The designation *tna* stands for the gene encoding tryptophanase from *E. coli*, an enzyme which converts tryptophan to indole (Stewart *et al., J. Bacteriol.* (1986) *166*:217-223).

The *pig* designation is used for the encoding sequence to the protein for indigo production from *Rhodococcus*, which produces indigo from indole (Hart *et al., J. Gen. Microbiol.* (1990) *136*:1357-1363). Both *tna* and *pig* were obtained by PCR. Tryptophanase is responsible for the conversion of tryptophan to indole, while the second gene (*pig*) encodes an indole oxygenase enzyme responsible for the conversion of indole to indoxyl. Both these bacterial genes were utilized in their native form.

The tobacco SSU transit peptide encoding DNA sequences were fused onto the amino terminal region of both the *tna* and *pig* indigo genes to effect localization of both the tryptophanase and indole oxygenase proteins to the fiber cell plastid. These are the same exact gene fusions that were made for the plastid-directed proteins for melanin production in construct 5148. The *tna* and *pig* gene products were targeted to the fiber cell plastid as that is the primary site of tryptophan synthesis.

D7

As with the melanin genes, the plastid-directed *tna* and *pig* genes were placed in the fiber-specific 4-4 promoter cassette and these chimeric genes were subsequently inserted into a binary plasmid to create plasmid pCGN5616. A block diagram of plasmid pCGN5616 is shown in Figure 8.--.

On page 41, after line 26 (the last line on this page), insert the following:

--Example 9

Expression of Transgenic Pigment Synthesis Genes

Melanin

Plants that exhibited resistance to the kanamycin selectable marker via a leaf assay and corresponding Western analysis were considered transformed. Transgenic fiber was collected from individual plant transformants at different stages of fiber development and analyzed in two ways. One was to analyze fiber at a single developmental time point for each transgenic cotton plant to compare tyrosinase expression between transgenic events. The other was to screen developing fiber from selected plants to analyze the timing of tyrosinase expression under the control of the fiber-specific 4-4, by the Western blots using antisera prepared against purified tyrosinase protein.

D8

For the plastid-targeted construct pCGN5148, 9 of 13 events screened for tyrosinase expression were positive, while 13 of the 16 transformed vacuolar-targeted construct pCGN5149 events which were screened were positive. Expression level in the fiber in tyrosinase positive plants is approximately 0.1-0.5% fiber cell protein. Clearly, the cotton fiber cells comprising the DNA color constructs DNA produce the necessary proteins required for synthesis of a pigment.

Visually, the lint from the tyrosinase positive events exhibits color to varying degrees, while plants that do not express the enzyme do not exhibit any color. Colorimeter measurements of cotton fiber taken from control Coker 130 plants and plants from various events transformed with pCGN5148 are provided in Figures 9 and 10, respectively.

Fiber from pCGN5148 (plastid-directed) plants demonstrates a bluish-green color phenotype. One event, 5148-50-2-1 included cotton fiber cells (linters) which were colored

D

and which had a negative a* value of less than -8.0, as measured on the L*a*b* color space. Coker 130 cotton fiber cells do not typically demonstrate a negative a* value.

These colored cotton cells also had a color located on the L*C*h* color space with a relatively high hue angle value, h, of greater than 135°. Normal Coker 130 fibers have a similar value which is not greater than about 90° as measured by this method.

Results of colorimeter measurements of cotton fiber taken from plants transformed with pCGN5149 are provided in Figure 11. Fiber from plants expressing tyrosinase from construct pCGN5149 (vacuolar-targeted) tends to have a light brown phenotype.

Indigo

Resistance to the kanamycin selectable marker via leaf assay and Western analysis was again the criterion for designating a plant as transformed by pCGN5616. Transgenic fiber was collected from individual plant transformants at different stages of fiber development. The transgenic developing fiber is screened from selected plants to analyze the timing of *tna* and *pig* gene expression under the control of the fiber-specific 4-4 promoter and fiber is also analyzed at a single developmental time point for each transgenic cotton plant for comparison of both tryptophanase and indole oxygenase expression between transgenic events, by using Western blots with antisera prepared against the tryptophanase and indole oxygenase proteins.

For the indigo events, 15 of 24 screened plants were positive for expression of both the tryptophanase and indole oxygenase enzymes. Expression levels in the fiber of these proteins is between 0.05-0.5% fiber cell protein. Approximately half of these transformants are expressing both genes in the fiber resulting in a very faint light blue color phenotype. Visually, there is a faint blue color in the majority of these positive events, particularly in 20-30 dpa fiber in the unopened boll. Results of colorimeter measurements of cotton fiber taken from various events of plants transformed with pCGN5616 are provided in Figure 12. Many of these events had relatively low a* values (less than 2) with elevated b* values (greater than 10), as measured on the L*a*b* color space. Similarly, several 5149 also measured with an a* value less than 2 while maintaining a b* value greater than 10.

BC Cotton

Colorimeter measurements taken on naturally colored fiber from four separate BC cotton lines is provided in Figure 13.

D8

The above results demonstrate that the color phenotype of a transgenic cotton fiber cell can be altered by expressing pigment synthesis genes. The transgenic cotton fiber cells include both a pigment synthesizing protein, and pigment produced by the pigment synthesizing protein. As shown from the results of Figures 9 through 13, expression of a pigment gene of interest can result in cotton fiber cells in which the synthesis of pigments combined with appropriate targeting sequences results in modification of color phenotype in the selected plant tissue, yielding colored cotton fiber by expression from a genetically engineered construct.--.

Please insert the paper copy of the sequence listing that was submitted on September 7, 1999 into the specification as pages 43-70, and renumber the following pages accordingly.

IN THE CLAIMS

Sub E1

D9

1. (Thrice Amended) A DNA sequence comprising as operably joined components in the direction of transcription, a cotton fiber transcriptional factor functional in a cotton fiber cell and an open reading frame encoding a protein in a pigment biosynthesis pathway, wherein said transcriptional factor is selected from [cotton 4-4 and cotton *rac* promoter sequences and is obtained by the method of probing a genomic library derived from a plant fiber tissue with up to the full length of a probe derived from the cDNA sequence shown in SEQ ID NO:1 or SEQ ID NO:7 or SEQ ID NO: 12 or SEQ ID NO: 15 or SEQ ID NO: 16 and isolating a nucleotide

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